to detect hybrid particles. Although the RNA of dependent virus often replicated to a level about 10 per cent of that of supporting virus, maturation of intact dependent virus within heterologous capsid was much less efficient. This could be due to a better fit between capsomeres and homologous RNA or it could be due to a spatial relationship between capsomere protein synthesis and the viral RNA which serves both as the template for such synthesis and as a nucleus for capsomere aggregation into capsid. A definite sequence of replication of viral RNA and protein may well be required for efficient encapsidation of RNA. This would explain why heterologous viral RNA superinfecting cells late after infection by another virus is not enclosed within the capsid protein being synthesized by the first virus (Table 1). Compartmentalization of virus within the cell might also explain this failure.

Finally, it is obvious that hybrid virus of this type offers a simple tool to determine whether virus host range and tissue tropism is determined by virus capsid affinity for cell receptors, as has been suggested earlier.

**Summary.**—Under certain in vivo conditions of double infection, the protein capsid of a replicating type 2 poliovirus or B1 Coxsackie virus enclosed the RNA of type 1 poliovirus to form millions of mature “hybrid” virus particles whose immunologic phenotype differed from their genotype.

* Supported by a grant from the National Science Foundation.

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**THE NATURE OF IRON IN FERREDOXIN**

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Ferredoxin is an electron-transport protein which contains seven atoms of non-heme iron and six or seven atoms of “labile” sulfide and has a molecular weight of 6,000.1 The iron of ferredoxin probably plays an important role in the electron-transfer function of the molecule. This paper describes Mössbauer effect, magnetic susceptibility, and chemical studies on ferredoxin which indicate that all of the iron is ferric in a strong ligand field.

**Methods.**—Clostridium pasteurianum was grown on sucrose with nitrogen gas as the sole nitrogen source as described by Carnahan and Castle.4 Ferredoxin was prepared according to the procedure of Mortenson, Valentine, and Carnahan1 as modified by Tagawa and Arnon.6 Ferredoxin was assayed by measuring its stimulation of acetyl phosphate formation from pyruvate.1

**Protein determination:** Protein was determined either spectrophotometrically or by dry weight. For dry weight determinations a salt-free solution of ferredoxin was lyophilized to dryness and
weighed. For routine work the optical density at 388 \( \text{m} \mu \) was used as a measure of protein concentration. The \( K \) value of ferredoxin at 388 \( \text{m} \mu \) is 3.75. Ferredoxin concentrations were determined optically with the same results either in 0.05 \( M \) Tris, pH 6.7, or in distilled water.

**Determination of total iron, ferric iron, ferrous iron, and inorganic sulfide:** Total iron content was determined by the \( \text{p}-\text{phenanthroline method}.^4 \) The sample was prepared by treating 1.5 ml of a solution containing 0.050–0.700 \( \mu \)moles of iron with 0.1 ml of 1 \( N \) HCl and heating at 80° for 10 min. This treatment bleaches the ferredoxin and is sufficient to remove all iron from the protein.

The chemical determination of the ferrous iron content of ferredoxin was based on the method of Fry, Lazzarini, and San Pietro in which the ferrous-\( \text{p}-\text{phenanthroline complex was measured after blocking the reducing groups of ferredoxin, viz., cysteine sulphydryl and "labile" sulfide, with \( \text{p-} \)chloromercuribenzenesulfonic acid (PCMS) or sodium \( \text{p-} \)[(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxycetate (sodium mersaly)]. Ferric iron was determined as the additional ferrous iron formed by reduction with hydroxylamine or mercaptoacetic acid. All determinations were performed in an anaerobic cuvette, and all solutions were deoxygenated by purging with nitrogen for 20 min prior to use. All manipulations were performed in a nitrogen atmosphere in a polyethylene bag. Addition of PCMS (2.50 \( \mu \)moles) in 1 ml of water to ferredoxin (0.017–0.033 \( \mu \)moles) in 1 ml of water bleached the ferredoxin to a colorless solution immediately. To this solution were added 1 ml of 0.2 \( M \) biphthalate buffer, pH 4.0, and 2 ml of a 0.3% \( \text{p}-\text{phenanthroline solution, and the contents were mixed.} \) The optical density of the ferrous-\( \text{p}-\text{phenanthroline complex was read at 512} \mu \) within 3 min. The ferric content was obtained on a duplicate solution by adding 0.4 ml of 5% \( \text{NH}_2\text{OH-HCl} \) and swirling for 10–20 sec before the addition of the \( \text{p}-\text{phenanthroline.} \) The increase in ferric content on addition of the reducing agent \( \text{NH}_2\text{OH-HCl} \) was taken as a measure of the ferric content of the PCMS-ferredoxin. In all cases the ferric plus ferrous content agreed with the total iron content to within 5%.

The inorganic sulfide content was determined by an adaptation of the method of Fogo and Popowsky. A

**Preparation of ferredoxin-Fe\(^{57}\) for Mössbauer studies:** Lovenberg, Buchanan, and Rabinowitz have shown that all iron atoms of ferredoxin are exchangeable with ferric iron after treatment of the protein with sodium mersaly, and that active ferredoxin can be regenerated by the addition of mercaptoethanol. We have used this procedure to incorporate Fe\(^{57}\) into ferredoxin. A comparison of the properties of native ferredoxin and reconstituted ferredoxin is shown in Table 1. The protein was lyophilized to dryness for determination of the Mössbauer spectrum.

**Magnetic susceptibility determinations:** The solid-state magnetic susceptibility of ferredoxin was determined by the conventional Faraday method using 3-mg samples of lyophilized ferredoxin. Field dependence measurements were made to eliminate the possibility of contamination by ferromagnetic impurities.

The magnetic susceptibility of ferredoxin in solution was determined by a nuclear magnetic resonance (NMR) technique in which the shift of the proton resonance line of an inert reference due to a paramagnetic substance is given by \( \Delta H/H = (2\pi/3)\Delta \), where \( \Delta \) is the change in volume susceptibility. All measurements were made at 60 me with a Varian HR-60 NMR spectrometer. Samples consisting of 30–80 mg of ferredoxin per ml of 93% \( D_2O \) with 3% tetramethylamonium chloride added as internal reference were contained in standard 5-mm NMR spinning tubes. As an external reference, sealed 1.5-mm capillary tubes containing 30% tetramethylamonium chloride in \( D_2O \) were placed inside the tubes containing the ferredoxin. Blanks containing no ferredoxin were measured after each sample. The shift due to the paramagnetism of the ferredoxin was obtained by subtraction of the internal-external reference shift of the blank from that of the ferredoxin-containing sample. Line separations were measured by the conven-

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<th>Table 1. Properties of Fe(^{57})-Ferredoxin</th>
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tional side band technique using a Hewlett Packard 200 AB audio oscillator and a Hewlett Packard 522 B electronic counter.

Mössbauer effect: Apparatus and experimental procedure for the Mössbauer effect have been described elsewhere\textsuperscript{11} and will not be detailed here.

Results.—Magnetic susceptibility: Magnetic susceptibility studies were carried out on solid ferredoxin and on aqueous solutions of ferredoxin to elucidate the oxidation states and ligand field environments of the seven iron atoms of the molecule. Four possibilities were considered most likely: weak-field Fe\textsuperscript{(III)}, strong-field Fe\textsuperscript{(III)}, weak-field Fe\textsuperscript{(II)}, and strong-field Fe\textsuperscript{(II)}. Ranges of magnetic moments exhibited by octahedral coordination compounds of iron in these oxidation states and ligand field environments are, respectively, 5.7–6.0, 2.0–2.5, 5.1–5.7, and 0 Bohr magnetons.\textsuperscript{12} If, alternatively, the iron of ferredoxin is tetrahedral rather than octahedral, the only substantial change from the ranges quoted above would be that the magnetic moment for strong-field, tetrahedral Fe\textsuperscript{(II)} would be about 3.0 Bohr magnetons.

The magnetic susceptibilities in solution of five different preparations of ferredoxin were measured as described under Methods. Pascal's constants\textsuperscript{9} were used to compute a value of $-2.56 \times 10^{-3}$ for the molecular diamagnetism of ferredoxin. With this diamagnetic correction, values were calculated for the molar paramagnetic susceptibilities, $\chi_0$, which were converted into average effective magnetic moments for iron by means of the equation:

$$\chi_0 = \frac{N \beta^2}{3kT} \sum n_\mu_i^2.$$ 

The results are given in Table 2. In the above equation, $n_i$ is the number of iron atoms possessing the magnetic moment $\mu_i$. The summation is over nonequivalent groups of iron atoms; for ferredoxin $\sum n_i = 7$. $\beta$, $N$, $k$, and $T$ are, respectively, the Bohr magneton, Avogadro number, Boltzmann constant, and absolute temperature.

If the seven iron atoms of ferredoxin are considered to be equivalent with respect to oxidation state and ligand field splitting (strong-field or weak-field), the average magnetic moment per iron atom for the five susceptibility measurements in solution (Table 2) is 2.0 $\pm$ 0.2 Bohr magnetons. Initial determinations of the solid-state magnetic susceptibility of ferredoxin were made with compacted samples; the values obtained were erratic and the recovered ferredoxin was insoluble. Compaction apparently causes irreversible changes in configuration in ferredoxin, probably involving alterations in iron coordination and/or oxidation states. Solid-state susceptibility determinations on uncompacted samples yielded a magnetic moment of 2.3 Bohr magnetons per iron atom, and the recovered ferredoxin was unaltered. Thus, solid-state and solution susceptibility determinations concur in assigning to ferredoxin an average magnetic moment per iron atom of 2.0–2.3 Bohr magnetons, a value that fits nicely into the range of strong-field ferric coordination compounds.\textsuperscript{12}

The solution value of 2.0 $\pm$ 0.2 Bohr magnetons for the effective magnetic
moment of each of the seven iron atoms of ferredoxin was obtained under the assumption that the seven iron atoms are equivalent with respect to oxidation state and strength of ligand field. Three combinations of strong-field Fe(III) and strong-field Fe(II) give computed susceptibilities that are considered to be within the limit of error of the experimental determination. These are $6 \text{Fe(III)} + 1 \text{Fe(II)}, 5 \text{Fe(III)} + 2 \text{Fe(II)},$ and $4 \text{Fe(III)} + 3 \text{Fe(II)},$ all strong-field. These three combinations appear to be less probable on the basis of results of the Mössbauer and chemical chelation studies than the situation in which all seven iron atoms of ferredoxin are strong-field Fe(III). Other possible combinations of oxidation states and ligand field strengths give calculated magnetic susceptibilities which differ widely from the experimental value.

Mössbauer spectra: In principle, Mössbauer spectra of coordinately bound iron permit determination of the number and relative populations of nonequivalent environments and bonding situations of iron. In addition, isomer shifts and quadrupole splittings reflect environment and valence state of coordinately bound iron. Unfortunately, the isomer shift and quadrupole coupling constant (quadrupole splitting) of the Mössbauer effect are somewhat akin to the chemical shift and spin-spin splitting of nuclear magnetic resonance spectroscopy in the sense that the relationships of the Mössbauer parameters to the valence states and environments of coordinately bound iron must, to a large extent, be established empirically.

Of the two Mössbauer parameters, the quadrupole coupling constant is more susceptible to theoretical analysis. The quadrupole coupling constant of iron depends on the product of the nuclear quadrupole moment of excited Fe$^{57}$ (I = 3/2) and the electric field gradient in which the iron nucleus resides. For totally symmetrical electron and charge distributions about the iron nucleus, the quadrupole coupling constant would vanish identically because of absence of an electric field gradient. For isolated Fe$^{57}$ as weak-field Fe(III) and strong-field Fe(II), the electric field gradient vanishes, and the quadrupole coupling constant is zero. Strong-field Fe(III) characteristically exhibits quadrupole splittings in the range 0.5–1.0 mm/sec, and weak-field Fe(II) exhibits splittings in the range of 2–3 mm/sec. Unfortunately, as with most empirical correlations, these ranges can be violated in particular instances and in fact appear at present to be most applicable to the more nearly ionic metal-ligand bonding situations. In the more covalent situations, the above considerations appear to be less restrictive.

Mössbauer spectra at 298° and 77°K of iron in ferredoxin enriched to the extent of 80 per cent in Fe$^{57}$ are shown in Figure 1. The spectrum at 298°K con-

![Fi. 1.—Fe$^{57}$ Mössbauer spectra of ferredoxin at 77° and 298°K. A possible decomposition of the 77°K spectrum into two overlapping doublets is indicated by the two curves without data circles.](image-url)
sists of two resonances of equal intensity separated by 0.70 mm/sec. The 77°C K spectrum differs significantly from the 298°C K spectrum only in that the spacing between the resonances is increased to 0.93 mm/sec.11 The existence of two resonances is attributed to quadrupole splitting of Fe57. The isomer shift varies from 0.42 to 0.52 mm/sec between 298°C and 77°C K. Upon close inspection of a number of Mössbauer spectra obtained at 298°C, 195°C, and 77°C K, it was concluded that the spectrum of ferredoxin actually consists of two overlapping doublets which have very similar isomer shifts and quadrupole coupling constants. The two doublets are so closely coincident that it is difficult to make a quantitative estimate of their relative integrated intensities and, consequently, the number of iron atoms contributing to the resonances of the two groups. A possible decomposition of the Fe57 spectrum of ferredoxin into the two suggested component doublets is, however, indicated in Figure 1.

The Mössbauer spectra indicate then that the seven iron nuclei of ferredoxin are distributed into at least two (and probably only two) nonequivalent but closely similar environments. The quadrupole splittings of 0.7 mm/sec (at 298°C K) are most consistent with Fe(III) iron in a strong ligand field. In such a bonding situation, the iron atoms would possess only single unpaired electrons and would give rise to magnetic susceptibilities of 2.0–2.5 Bohr magnetons.12 The observed isomer shift of 0.42 mm/sec in ferredoxin is not inconsistent with this conclusion. Combinations of strong-field Fe(III) and strong-field Fe(II) such as those considered in the section on magnetic susceptibility are not completely incompatible with the Mössbauer spectra, but it would be an improbable coincidence if both the isomer shifts and the quadrupole coupling constants of these two types of iron were nearly identical in ferredoxin. The Mössbauer results, however, cannot in themselves be taken as definitive but must be considered together with the results of chemical and other physical studies in elucidating the nature of coordinately bound iron.

Chemical studies: The difficulty of determining by chemical methods the valence of iron in nonheme proteins has been recognized recently.8, 7, 15 Fry and San Pietro earlier reported that o-phenanthroline forms an Fe(II) complex with both of the iron atoms of photosynthetic pyridine nucleotide reductase (PPNR).15 Their more recent experiments using PCMS to block the reducing groups of the protein indicate, however, that the two iron atoms are Fe(III).7 Lovenberg, Buchanan, and Rabinowitz have demonstrated that o-phenanthroline removes the seven iron atoms from ferredoxin as Fe(II), but they point out that any Fe(III) in the molecule could be reduced by cysteine sulfhydryl and/or "labile" sulfide on removal from the protein.8 In support of this they showed that ferredoxin reduces added Fe(III) to Fe(II).

In our experiments an excess of PCMS was added to block the reducing groups in an attempt to obtain an accurate measure of the Fe(III) and Fe(II) content of ferredoxin. The results of these experiments are shown in Table 3. These results show that after reaction with PCMS the seven iron atoms of ferredoxin exist as two Fe(III) and five Fe(II) atoms. There is no evidence, however, that reduction of Fe(III) to Fe(II) does not occur even in the presence of PCMS, and it is our contention that such a reduction of five of the seven iron atoms does occur since the magnetic susceptibility and Mössbauer spectra strongly indicate that all seven
iron atoms of ferredoxin are Fe(III). In themselves the o-phenanthroline results show only that (1) the seven iron atoms of ferredoxin are distributed into two structurally nonequivalent groups of two and five, and (2) there are at least two Fe(III) atoms in ferredoxin.

Discussion.—The conclusion drawn from these chemical and physical studies is that all seven iron atoms of ferredoxin are Fe(III) and that they are distributed into two structurally nonequivalent strong ligand field environments of populations two and five. It is tempting to ascribe a special significance to the numerical equivalence of iron atoms (seven), cysteine residues (seven), and “labile” sulfur atoms (six or seven) and to implicate these units in the electron transport function of ferredoxin. Indeed, evidence that each iron is bonded to a cysteine sulfur has been reported by Lovenberg, Buchanan, and Rabinowits.

It seems likely that iron is involved in the electron transport and undergoes valence change during the oxidation and reduction of the protein. An analogous situation has been observed with PPNR where the oxidized protein apparently contains two Fe(III) atoms, and the reduced protein contains one Fe(III) and one Fe(II). Since PPNR contains two “labile” sulfur atoms as well as two nonheme iron atoms, the electron transport sites or regions of ferredoxin and PPNR may have close structural similarities.

The construction of detailed molecular models in the absence of X-ray results is fraught with dangers. However, a model for ferredoxin can be set forth that rationalizes a good deal of chemical and physical data and has some interesting implications with respect to electron transport. It is to be considered only a working hypothesis that is to be rejected or refined in the light of further structural studies, particularly X-ray, on ferredoxin.

The model for the active site of ferredoxin that we wish to propose is shown in Figure 2. It is intended to suggest that the seven iron atoms of ferredoxin are arranged linearly, that they are bonded to each other via sulfur bridges furnished by the seven cysteine residues and six (or seven) inorganic sulfide atoms, and that they are held to the protein by the sulfur atoms of the cysteine residues. The iron is intended to be shown in Figure 2 as tetrahedral, although it could equally well be written (in so far as our results are concerned) as square planar or octahedral with similar sulfur bridging and water or carboxyl groups occupying the fifth and sixth coordination sites. The two terminal iron atoms in this model are structurally different from the five interior iron atoms, which is compatible with the chemical behavior and Mössbauer spectrum of ferredoxin. This model is also consistent with the rapid loss of the “labile” sulfide as H2S on mild acidification and with the evidence that the iron atoms are bound to the protein by cysteine sulfurs.

The similarity between the postulated active site of ferredoxin and the X-ray established structures of the red Roussin compound (Fig. 3) and the polymeric
KFeS₂⁺ (Fig. 4) is obvious. In both of these compounds the Fe-Fe distance is 2.7 Å which leads to a strong covalent interaction between the iron atoms. Such an interaction between the iron atoms of ferredoxin should lead to facile electron transport down the linear chain pictured in Figure 2. As further precedent for this model of ferredoxin, Jensen found that the product of the reaction between Ni(II) and ethyl mercaptan is a linear polymeric substance which consists of one nickel atom and two ethylmercapto groups as the repeating unit. The nickel atoms are in a square planar (or tetrahedral) configuration, and adjacent nickel atoms are connected by sulfur bridges of two ethylmercapto groups. Similar but nonpolymeric metal-sulfur bridge structures have been found by Wrathall and Busch for Ni(II), Pd(II), and Pt(II) with a variety of SH-containing ligands.

As suggested earlier, there may be common structural elements, at least in so far as iron is concerned, in PPNR and ferredoxin. If, then, our model for ferredoxin is correct, a structure for PPNR in which the two iron atoms are connected by sulfur bridges and bound to the protein via cysteine residues would not be unreasonable. In light of this model it is of interest to consider the imidazole-pump model for the cytochromes discussed by Urry and Eyring in which electron transport between iron atoms of proximal cytochrome molecules is accomplished by imidazole components of histidine residues acting as bridging groups.

Summary.—(1) The magnetic susceptibility and Mössbauer spectra of ferredoxin indicate that all seven iron atoms are strong-field ferric but that they exist in two structurally nonequivalent environments. The chemical studies on ferredoxin indicate that the iron atoms are distributed into two structurally nonequivalent states of populations of two and five and that there are at least two ferric atoms in ferredoxin.

(2) A model for the active site of ferredoxin is proposed in which the seven iron atoms are arranged linearly and are bound together via sulfur bridges furnished by the seven cysteine residues and six inorganic sulfide atoms.

* Contribution no. 964.
ON MEMORY AND RECALL*

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The subject matter of this paper is a hypothetical biological process on which the capability of the central nervous system to record and to recall a sensory experience might conceivably be based. It may be open to doubt whether one knows enough about the living cell to be able to say anything with reasonable assurance about the molecular processes that the brain employs. Still, with luck, one might perhaps guess correctly the general nature of these processes. To what extent we may have succeeded in doing so remains to be seen.

The Efficacy of a Synapse Bridging Two Neurons.—Our neural network models involve excitatory neurons and inhibitory neurons (of the kind which exert a postsynaptic inhibitory effect).

Let us consider an excitatory neuron which contacts through a synapse another neuron. If such an excitatory neuron sends a volley of nerve impulses to this synapse, then a certain quantity of an excitatory "transmitter substance" is released in the vicinity of the presynaptic membrane which diffuses across a gap—the synaptic cleft—into the postsynaptic neuron and raises the level of excitation of that neuron by a certain amount. We shall designate this excitatory transmitter substance as "acetylcholine" (in quotes). The "acetylcholine" which diffuses into the postsynaptic neuron is destroyed, in the vicinity of the postsynaptic membrane, by an enzyme which we shall designate as "choline esterase."

The rate at which "acetylcholine" is released in the vicinity of the presynaptic membrane is a function of the frequency of the nerve impulses which reach the synapse, and we shall designate this rate as the "signal intensity." For the sake of simplicity, we shall assume that the signal intensity is for all synapses the same function of the frequency of the nerve impulses which are fed into the synapse.

The rate at which "acetylcholine" is destroyed in the postsynaptic neuron is proportional to the product of the concentration of "acetylcholine" and the concentration of the enzyme "choline esterase" in the vicinity of the postsynaptic